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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Application of :
Kristian BERG et al. :
Serial No. 09/524,454 : Examiner: G.R. Ewoldt
Filed: March 10, 2000 : Group Art Unit: 1644
For: METHOD OF EXPRESSING ANTIGENS ON THE SURFACE OF ANTIGEN PRESENTING CELLS BY PHOTOCHEMICAL INTERNALIZATION :

DECLARATION UNDER RULE 132

Commissioner of Patents
and Trademarks
Washington, D.C. 20231

I, Anders Høgset, a Norwegian citizen of Treskeveien 32A, 0681, Oslo, Norway,

declare as follows:

1. I am an inventor on the present application. I have reviewed the Office Action dated 18 June 2002 which issued on the above application, wherein the Examiner raised objections under 35 U.S.C. 112, first paragraph, that the specification does not enable performance of the invention as claimed.

2. To demonstrate that the invention as claimed may be put into practice in accordance with the specification, I provide details of experiments which have been conducted in my laboratory which show that Example 2 in the specification does indeed show that T lymphocytes identify antigen expressed on the surface of the cells which has been internalized by the photochemical internalization method of the invention and that the process of photochemical internalization (PCI) may be regulated such that the level of cell killing (resulting from PDT) is controlled. The experiments which have been conducted are described in the following paragraphs.

Surface expression of MART-1 after PCI

3. In paragraph 5 of the Official Action the Examiner asserts that Example 2 fails to show cell surface expression of the MART-1 peptide. The following experiment shows that MART-1 specific CTLs exhibit higher cytotoxicity to cells to which MART-1 has been applied and which have been subject to photochemical internalization.

4. In the experiment the same methods were followed as described in Example 2 except that the experiments were performed with or without the MART-1 peptide. The experiment was conducted in the presence of CTLs and photosensitizer and illumination was performed for various times. The results are shown in Figure 1 in Annex 1. It will be seen from this Figure that virtually no CTL-dependent cell killing occurs without the MART-1 peptide, regardless of whether the cells are illuminated or not. Addition of the MART-1 peptide induces a small level of cell killing (about 3.5%) without illumination, but illumination increases the cell killing substantially (about 4-fold in this experiment). In view of the selectively of CTL for MART-1 peptide appropriately processed and presented on the surface of the cell, this illustrates that photochemical treatment results in MART-1 internalization, processing and presentation on the surface of the cells in a form such that immune effector T cells are able to recognize and eliminate those cells.

Cell death during photochemical internalization

5. Whether or not cell death results after photochemical treatment is principally dependent on two factors. Firstly the amount of toxic substances generated by the photosensitizing compounds on exposure to light and secondly, the presence and toxicity of molecules which are internalized during this process. The level of toxic substances which are generated may be controlled by the selection of the photosensitizer to be used, the dose of that photosensitizer, but most crucially, the time of illumination which leads to increasing levels of the toxic substances. The

second aspect, namely the toxicity resulting from the molecules which are introduced may be readily controlled by selecting an appropriate toxic or non-toxic molecule for transfer, depending on the desired end use.

6. The following experiments illustrate that by an appropriate selection of illumination time *in vitro* or *in vivo*, cell survival may be maximized whilst retaining uptake of the transfer molecule.

7. In the *in vitro* experiment, HCT 116 cells were treated with aluminium phthalocyanine disulfonate ($AlPcS_{2a}$) overnight, washed for 4 hours and incubated with a pEGFP-N1/polylysine complex for 4 hours. The cells were then illuminated with red light for various times and 48 hours later cells expressing enhanced green fluorescent protein (EGFP) (transfected cells) were scored by flow cytometry. Cell survival was measured by the MTT assay. The results are shown in Figure 2 in Annex 1.

8. Figure 2 clearly shows that both the cell survival and the PCI effect (ie. the transfection efficiency in this case) can be tightly controlled by varying the light dose. As the light dose increases, cell survival decreases (the PDT effect), but it can be seen, e.g. at 4 minutes illumination, that there is a very substantial PCI-effect (about a 15-fold increase in transfection) under conditions in which about 85% of the cells survive the light treatment. Thus it will be seen that PCI effects occur without substantial cell killing.

9. In the *in vivo* experiment, Balb/c (*nu/nu*) mice with subcutaneous WiDr tumours were injected intraperitoneally with 10mg/kg $AlPcS_{2a}$, 42 hours before direct intratumoural injection of gelonin at 50mg/tumour. Six hours after injection of gelonin the mice were exposed to 135 J/cm^2 of light. 6-9 animals per group were subject to either (i) gelonin treatment alone, (ii) $AlPcS_{2a}$ treatment and light or (iii) $AlPcS_{2a}$ and gelonin and light, and the tumour volume examined as a function of time after light treatment. The results are shown in Figure 3 in Annex 1.

10. Figure 3 shows that the use of the photosensitizer and light alone was unable to kill all tumour cells. Whilst the tumour growth initially halted, growth was once again observed after about 20 days. In contrast, when the toxin gelonin was also added in addition to the photochemical treatment there was a dramatic effect on tumour growth which showed that the toxin which was internalized was responsible for further death of cells not killed by the treatment using the photosensitizer alone. In these group of animals about 70% of the animals were totally cured of their tumours. The curves to the left of the Figure marked "gelonin" represent the results of control experiments and include experiments in which gelonin alone was used but found to have no effect without the photochemical treatment.

11. This clearly shows that in this experiment even though the experiment was biased towards achieving cell death, not all tumours cells which were subject to photochemical treatment were killed. However it is clear that a portion of cells which are not killed are still affected by the photochemical treatment. This is clear from the results using gelonin with the photosensitizer and light. For gelonin to be effective at causing further cell death it needs to be internalized (see the controls in which gelonin without photosensitizer had no effect). Thus the results show that photochemical treatment mediated internalization of gelonin into cells which had not been killed by the photosensitizer and light alone. These cells were then killed by the toxic action of gelonin. This thus shows that the tumour cells that are affected by photochemical treatment may be divided into two classes. Those cells which are subject to such high levels of toxic substances resulting from the activation of the photosensitizer by illumination (which are likely to be the cells closest to the source of illumination) that they were killed and those cells which survive and which internalize transfer molecules via photochemical internalisation, but which are then killed as a result of the toxic nature of the transfer molecule which they internalize.

12. These experiments thus show the variable effects that photochemical treatment has on cells, and that the effects are

controllable, particularly by manipulating the extent of illumination. Thus cell death can be readily manipulated and reduced as desired to favour the internalization effects without cell death.

13. The above experiments illustrate that internalization results in processing and presentation of the internalized molecule and that the level of cell killing during photochemical treatment can be readily minimized to favour photochemical internalization processes.

14. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Codes, and that such wilful false statements may jeopardize the validity of the application and any patent issuing thereon.

Anders Høgset ..... 13.11.02 .....

Anders Høgset Date

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ANNEX 1

Figure 1

CTL-dependent cell killing as a function of illumination time for cells incubated with or without the MART-1 peptide.

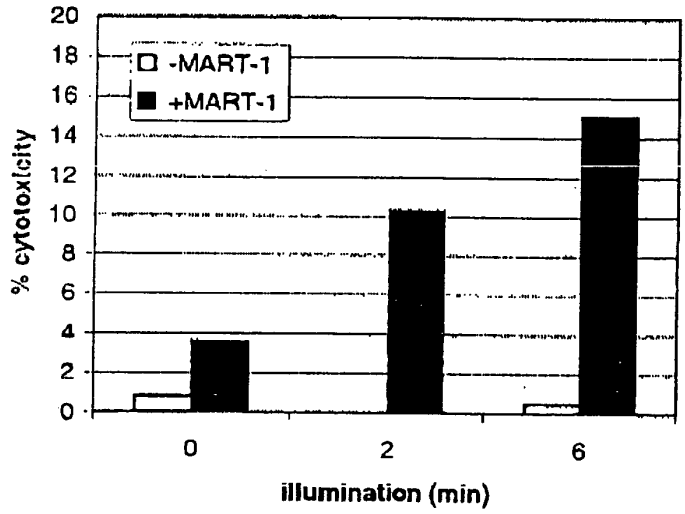

Figure 2

Transfection and cell survival as a function of light dose for cells subjected to PCI-mediated transfection with a plasmid/polylysine-complex.

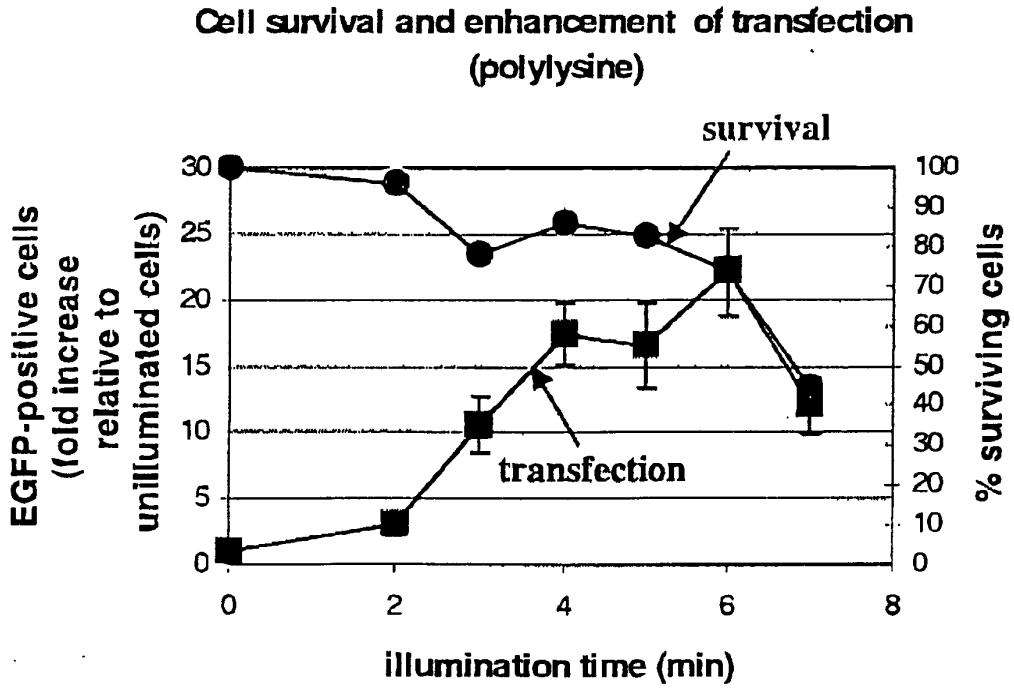

Figure 3

Treatment of subcutaneous WiDr tumours *in vivo* by photochemical internalization of gelonin.